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The action of pyrethroids on the voltage-sensitive calcium channel of *Paramecium tetraurelia*

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Abstract: Calcium regulation is an important event in synaptic transmission and neuronal function, which is governed by a very intricate signal transduction system which is not completely understood. Using a variety of pharmacological assays, we have characterized the action of deltamethrin on the ciliary voltage-sensitive calcium channel and on phospholipase C activity of *Paramecium tetraurelia* Sonneborn, an organism that does not possess a voltage-sensitive sodium channel. In fura-2 fluorometric assays, which examined whole cells and ciliary membrane vesicles enriched with calcium channels, deltamethrin stimulated Ca^{2+} uptake. We also determined that the phospholipase C activity of the ciliary membrane vesicles is regulated by the βγ-subunit from heterotrimeric G-proteins. Subsequent treatment with deltamethrin resulted in a substantial and highly significant increase in phospholipase C activity. These results provide evidence that the molecular mode of action of pyrethroids on the voltage-sensitive calcium channel is distinct from the action of this insecticide on the voltage-sensitive sodium channel and may be dependent, in part, upon an interaction with the βγ-subunit of heterotrimeric G-protein.

Keywords: pyrethroids; voltage-sensitive calcium channel; G-proteins; phospholipase C; *Paramecium tetraurelia*

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(*Received 25 August 1998; revised version received 30 November 1998; accepted 20 May 1999*)

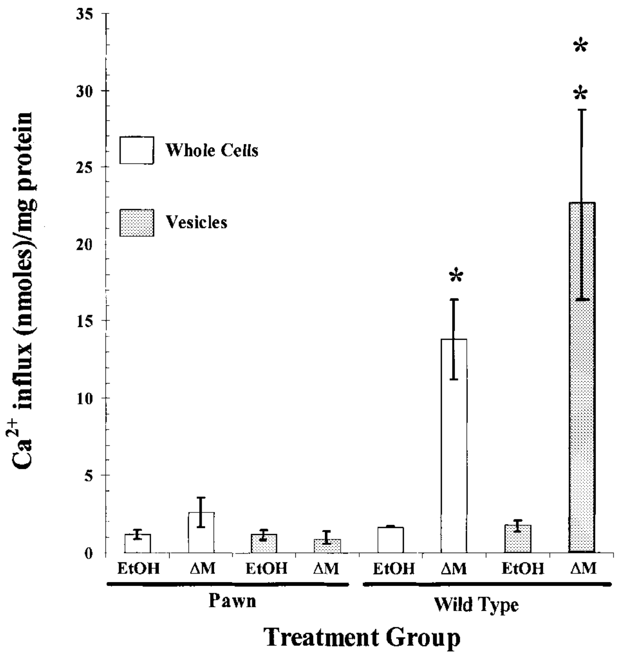

Figure 1. Fluorescent determination of the effect of 10^{-7} M deltamethrin (ΔM) on Ca^{2+} influx in whole cells and ciliary calcium-channel-containing membrane vesicles from *Paramecium tetraurelia.* (*) indicates that deltamethrin treatment is significantly different from ethanol control ($p < 0.05$). (**) indicates that deltamethrin treatment is significantly different from ethanol control ($p < 0.06$).

Type II pyrethroids, including deltamethrin, are toxic to *Paramecium tetraurelia* Sonneborn, an organism that does not possess a voltage-sensitive sodium channel. In behavioral bioassays, deltamethrin-treated cells exhibited an increase in backward swimming, a well-characterized avoidance response controlled by the voltage-sensitive calcium channel. The non-toxic 1*S* isomer of deltamethrin had no significant effect on either mortality or avoidance behavior of *Paramecium. Pawn* mutants, which lack a functional voltage-sensitive calcium channel, were likewise unaffected by deltamethrin. Intracellular recordings of whole cells showed that 10^{-9} M deltamethrin resulted in membrane destabilization, increased spontaneous action potentials, repetitive discharges, and membrane depolarization. Our initial findings established that the toxic effect of deltamethrin is structurally related, dose-dependent, and enhanced by depolarization, thus providing evidence that type II pyrethroids, specifically deltamethrin, act as potent calcium channel agonists in *P tetraurelia.*[1]

Figure 1 compares the effect of deltamethrin on Ca^{2+} influx in whole cells vs enriched calcium-channel-containing membrane vesicles as measured by fura-2 fluorometric assays. As expected, deltamethrin treatment of *pawn* mutants resulted in no significant increase in internal free [Ca^{2+}] in either whole cell or membrane vesicle assays. In similar experiments with wild-type cells, deltamethrin treatment increased internal free [Ca^{2+}] 8-fold in whole cells and 12-fold in the membrane vesicle preparations. These findings further substantiate our initial

contention that type II pyrethroids are calcium channel agonists in *P tetraurelia*, and show that the calcium-channel-containing vesicle preparation is an excellent system to examine the pharmacological events associated with the toxic action of deltamethrin on the voltage-sensitive calcium channel.

Recent evidence has shown that GTP-γ-S increased *Paramecium* backward swimming and prolonged the Ca^{2+} action potential.[2] Corroborating research conducted by Rossignol[3] implicated the β-subunit of heterotrimeric G-proteins as a target site for pyrethroids by showing that the binding of a photoreactive analogue of fenvalerate ([^{3}H]DeCAF) was increased by the addition GTP-γ-S. Also, calcium channel fusion protein research conducted by DeWaard *et al*[4] has shown that some isoforms of the voltage-sensitive calcium channel possess two distinct regions, AID and D2, that bind the $\beta\gamma$-subunit of G-proteins ($G_{\beta\gamma}$). Binding of $G_{\beta\gamma}$ to the D2 region resulted in an initial inhibition of the calcium channel. However, this initial inhibition could be overcome by a strong electrophysiological prepulse, which resulted in a facilitation mechanism that produced a 'willing' or conducting form of the calcium channel. Related studies conducted by Zamponi provided evidence implicating a protein-kinase-C-(PKC) dependent phosphoralytion in the D2 region that resulted in inhibition of the inactivation kinetics of the voltage-sensitive calcium channel (ie producing a conducting state).[5]

Given these preliminary data, we have examined various pharmacological pathways regulated by $G_{\beta\gamma}$.[6] We have found that membrane vesicles possess phosphodiesterase (PDE), protein kinase A (PKA), and phospholipase C activity (PLC). However, only PLC activity was modified by the addition of deltamethrin. Figure 2 shows the effects of various cofactors, inhibitors, and deltamethrin on PLC activity in calcium-channel-containing vesicles. Addition of exogenous $G_{\beta\gamma}$, an activator of PLC activity that functions at the β-subunit of this enzyme, significantly increased PLC activity in vesicles by 59% compared to control (Fig 2A). Addition of 10^{-7}M deltamethrin (ΔM) further stimulated $G_{\beta\gamma}$-PLC activity and resulted in an additional 10% increase that was highly significantly different compared to vesicles treated only with $G_{\beta\gamma}$. Platelet-derived growth factor (PDGF), a common PLC activator that functions by up-regulating PLC activity at the γ subunit of this enzyme, also stimulated PLC activity by 59% in vesicles. The addition of 10^{-7}M deltamethrin to PDGF-treated vesicles, however, failed to further stimulate PLC activity. Finally, PDGF up-regulation of PLC could be further increased by the addition of $G_{\beta\gamma}$ in the presence of 10^{-7}M deltamethrin. This increase, however, was not significantly different from deltamethrin plus $G_{\beta\gamma}$ treatment. Using U-73122, a strong PLC inhibitor that modifies the β-subunit of PLC responsible for $G_{\beta\gamma}$ activation,[7] we confirmed that the up-regulation of PLC by deltamethrin is via the $G_{\beta\gamma}$ (Fig 2B). Deltamethrin treatment of vesicles increased PLC activity by more than 2-fold. However, pretreatment of vesicles with U-73122 prior to the addition of deltamethrin reduced pyrethroid stimulation of PLC activity by a factor of three compared to deltamethrin treatment and was not significantly different from U-73122 treatment alone. These results show that deltamethrin enhances $G_{\beta\gamma}$ activation of PLC at the β-subunit of this enzyme. Using previous and current information, we have formulated a working hypothesis that deltamethrin, in part, increases Ca^{2+} flux via the voltage-sensitive calcium channels of *P tetraurelia* through an interaction with the $\beta\gamma$-subunit of heterotrimeric G-proteins.

A

Treatment	PLC Activity (pmol IP_3 min^{-1} mg^{-1} protein)	SE	Percent increase over control	P-value	
Ethanol control	11.5	1.9	–	–	
$G_{\beta\gamma}$	28.2	0.3	59.4	*0.007	
$G_{\beta\gamma}$ + 10^{-7}M ΔM	36.2	1.2	69.1	*0.003	0.021a
PDGF	27.8	1.8	58.6	*0.023	
PDGF + 10^{-7}M ΔM	29.0	0.7	60.8	*0.008	0.718b
$G_{\beta\gamma}$ + PDGF + 10^{-7}M ΔM	35.9	1.7	68.0	*0.005	0.057c

B

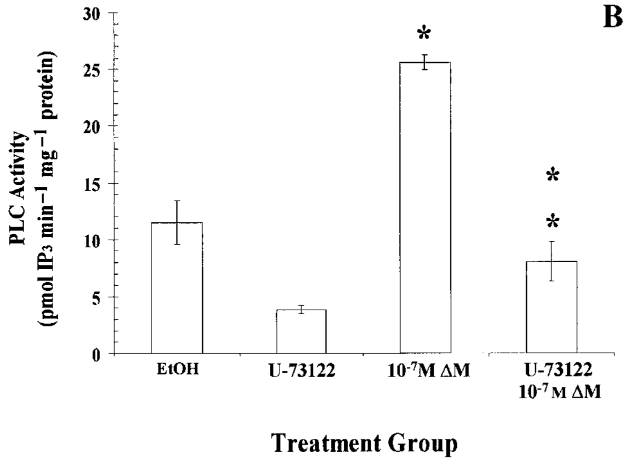

Figure 2. Effect of 10^{-7}M deltamethrin (ΔM) on PLC activity in calcium channel containing vesicles. **A**, effect of cofactors [10 nM G protein $\beta\gamma$-subunit ($G_{\beta\gamma}$), 10 nM platelet-derived growth factor (PDGF)] and deltamethrin on PLC activity in vesicles. (*) indicates a significant increase compared to ethanol control ($p<0.05$). (a) indicates a significant difference between $G_{\beta\gamma}$-PLC in the presence and absence of deltamethrin ($p<0.05$). (b) indicates no significant difference between PDGF-PLC activity in the presence and absence of deltamethrin ($p<0.05$). (c) indicates a significant difference between $G_{\beta\gamma}$+PDGF+Δ M-PLC activity compared to $G_{\beta\gamma}$ treatment alone ($p<0.06$). **B**, effect of 10^{-7}M U-73122 and ΔM on PLC activity. (*) indicates that deltamethrin treatment is significantly different from ethanol control ($p<0.05$). (**) indicates that U-73211 pretreatment is significantly different from deltamethrin treatment alone ($p<0.05$).

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In-vitro study on the effect of pesticides on neuronal activity

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Abstract: Experiments have been conducted to examine the effect of chronic administration of bromoxynil, fluroxipir and bensultap on the in-vitro seizure susceptibility (induced by 4-aminopyridine) and excitability of neocortical slices of rat brain. The treatment regimes were (A) administration of spray solution in place of drinking water for seven days, and (B) feeding wheat which had been sprayed at growth stage Feekes 9–10 and consumed four to six weeks after spraying. The latency of appearance of the first seizure was significantly increased by fluroxipir (B) bensultap (B) and bromoxynil (A&B). Fluroxipir (A&B) decreased the frequency of seizure, and fluroxipir (A) and bensultap (B) doubled the duration of seizures. Excitability following electrical stimulation of the corpus callosum was not significantly changed by any treatments. The changes in brain activity were not related to the residue levels of the pesticides in the rat brains. Our results suggest that these chemicals may alter the functional properties of neuronal network activity and neurotransmission in rat neocortex after environmental exposure.

Keywords: bensultap; bromoxynil; fluroxipir; neuronal activity; brain slice; neurotoxicity

1 INTRODUCTION

The human population is exposed to toxic effects of various environmental chemicals including pesticides. Therefore it is important to learn more about the mechanisms of low-level or accidental exposure to, and potential biological effects of these compounds. To date cumulative, irreversible effects such as carcinogenesis and neurodegeneration have received the most attention. Histological and biochemical investigations have shown that different pesticides may cause several types of abnormality in the immune or the reproductive system.[1] However, there is relatively little information concerning pesticide-induced functional changes in the central nervous system.

There is a relatively large body of evidence concerning the neurophysiological effects of organophosphorus compounds,[2–4] chlorinated hydrocarbons[5–7] and certain pyridine compounds in mammals.[8,9] These types of pesticide may disturb normal neurotransmission, induce convulsions,[7,10] inhibit enzymes[11,12] or they can induce apoptotic cell death.[13] It is well known that organophosphorus pesticides (eg chlorpyrifos) exert their effect through inhibition of acetylcholinesterase.[2–4] Another group, chlorinated hydrocarbons (eg lindane), increase the excitability of the nervous system, causing severe epileptic discharges or neurodegeneration. These compounds act on the $GABA_A$ receptor.[5–7] However, only sporadic data are available about the neurotoxic effects of other types of agrochemical in wide use.

In our experiments we have studied three frequently used agrochemicals. Neurophysiological effects of the compounds have not been adequately examined. The chronic, low-dose exposure of bromoxynil caused severe behavioural alterations in dogs and humans,[14] but there was no such detectable effect in rats. The other two pesticides studied have not been examined for neurophysiological and behavioural parameters. In our present neocortical slice experiments, the effects of chronic pesticide application on neuronal excitability as well as on seizure susceptibility were analysed to estimate many neurotoxic potential. The field responses evoked by electrical stimulation and spontaneous seizure activity developed in 4-aminopyridine (4-AP)-containing solution were analysed in detail in neocortical slices of control and pesticide-treated rats. We have chosen subtoxic exposure regimes that caused no mortality or illness.

2 EXPERIMENTAL METHODS AND MATERIALS

2.1 Pesticide exposure

The experiments were performed on adult Wistar rats of both sexes (120–160 g, LATI, Gödöllô). The pesticide-treated and control groups consisted of three and 12 animals respectively. The compounds were administered for seven days. The animals received the pesticides either dissolved in the drinking water at a defined dose (see below), or they were fed with wheat pre-treated with the pesticides. The concentrations in the drinking water corresponded to the standard crop-spraying concentration of each pesticide. Total volume

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Contract/grant sponsor: Hungarian Government; contract/grant number: OTKA 24059
(*Received 7 October 1998; revised version received 30 November 1998; accepted 20 May 1999*)